Application Note

Cell Analysis

Agilent

Trusted Answers

Real-Time, Label-Free Measurement of Natural Killer Cell Activity and Antibody-Dependent Cell-Mediated Cytotoxicity

Authors

Lauren Jachimowicz, PhD,
Peifang Ye, Ming Lei,
Jeffrey Li, and
Garret Guenther, PhD
Agilent Technologies, Inc.
Cell Analysis Division
San Diego, CA, USA

Abstract

The response of tumor cells (as target cells) to natural killer (NK) cell activity (as effector cells), in the presence or absence of immunoglobulin G isotype-specific antibody, was measured to determine if cell-mediated cytotoxicity (specifically antibody-dependent cell-mediated cytotoxicity (ADCC)) can be investigated using the Agilent xCELLigence system. It was shown that the addition of NK cells in suspension, over a monolayer of adherent tumor cells, does not produce impedance or Cell Index (CI) changes, because the NK cells do not come into contact with the biosensor.

However, the secretion of perforins and granzymes by these nonadherent NK cells did activate caspases inducing tumor cell apoptosis. Dysfunctional and dying tumor cells detached from the biosensor, reducing the number of viable and adherent cells on the biosensor surface. Our findings provided compelling evidence for how the Agilent xCELLigence system can be used for the dynamic real-time monitoring of cell-mediated cytotoxicity and the impact of specific antibodies.

Introduction

Cell-mediated cytotoxicity is an important means by which the body protects itself from pathogenic attacks, providing an essential defense mechanism against viruses, bacteria, or parasites, as well as transformed and dysfunctional cells. It also plays an important role in maintaining homeostasis of the immune system. Cell-mediated cytotoxicity specifically involves leukocytes that recognize and destroy other cells or invaded microbes. Two types of leukocyte-effector cells carry out this activity:

- Lymphoid cells known as cytotoxic T-lymphocytes, and NK cells
- Myeloid cells, such as macrophages, eosinophils, and neutrophils

The most important role of cytotoxic T-lymphocytes is the elimination of cells infected with viruses. In addition to expressing their own cell-specific peptides on their cell surface, infected cells also exhibit processed pathogen-specific antigens in association with the major histocompatibility complex (MHC) class I molecules. Virus-infected cells present these pathogen-specific antigens to cytotoxic T-lymphocytes, which are capable of distinguishing between self and nonself peptides and can mobilize an antiviral response accordingly.

Cytotoxic T-lymphocytes and NK cells act in a complementary way to protect the body because NK cells specifically recognize and kill cells that exhibit reduced (or have lost) MHC class I expression.

Significantly, tumor cells also commonly express reduced levels of MHC class I molecules, which provides the basis for an effective antitumor effect of NK cells. Cell-mediated cytotoxicity is a result of the following complex cell-cell interactions:

- Direct cell-cell interactions producing internalization and phagocytosis of infected cells, or pathogens by macrophages and neutrophils
- Secretion of cytokines, such as Fas ligand and tumor necrosis factors, by cytotoxic T-lymphocytes
- Release of granule proteins, such as perforin and granzymes, by both cytotoxic T-lymphocytes and NK cells
- Release of toxic molecules, such as reactive oxygen intermediates and lysosomal enzymes, by myeloid cells

Cellular degranulation is also facilitated by a process known as opsonization of the target cells by cross-linking antibodies. In this complex event, antibodies, using their Fab fragments, first specifically attach to antigens on the surface of the target cells to be killed. Opsonization confers the required spatial proximity between target and effector cells by specifically binding the Fc fragments of the target cells directly to the Fc receptors of the corresponding effector cells (cytotoxic T-lymphocytes and NK cells). The subsequent cross-linking of Fc receptors is an essential part of this immune response, leading to target cell death known as antibody-dependent cell-mediated cytotoxicity (ADCC).

The rationale for a new method to measure NK cell activity and additive antibody-dependent cytotoxicity

Monitoring and quantifying the lytic activity of effector cells, such as cytotoxic T-lymphocytes and NK cells, is important for defining physiological and pathophysiological states, response to infectious viral agents, and auto-immune reactions. These types of investigations are also essential for characterizing the lysis process itself and for identifying lysis mediators. The phenomenon of cell-mediated cytotoxicity through ADCC can be studied *in vitro*, using either fresh lymphocytes isolated from blood or NK cell lines as effectors. Appropriate *in vitro* targets include pathogen-infected eukaryotic cells and tumor cell lines.

The most common method for measuring cell-mediated cytotoxicity is the release assay based on the loss of target cell membrane integrity.[4] Up to four hours after effector cell addition resulting in target cell lysis, the radioactive release from target cells prelabeled with Chromium (^{51}Cr) or Indium (^{111}In) is measured, or the release of naturally occurring substances, such as lactate dehydrogenase (LDH), into the culture medium is assayed. Release of these substances serves as an indirect measure of the extent of cell damage due to effector cell-mediated target cell lysis.[4]

Alternative methods also include flow cytometry, enzyme-linked immunosorbent assay-based granzyme measurement, and morphometric analyses by microscopy.[5]

All these complex labor-intensive studies are endpoint assays with usually a single measurement over a period of hours. The use of radioactively labeled target cells is also inconvenient and may affect the cellular response being studied. In contrast, the Agilent xCELLigence system enables dynamic, label-free, and noninvasive analysis of cellular events in real time. This system is based on a microelectric sensor measuring impedance.[10]

The xCELLigence system

The xCELLigence product line includes the Agilent real-time cell analysis (RTCA) single plate (SP) and multiple plates (MP) instruments. Both the RTCA SP and MP consist of several components:

- RTCA for real-time data acquisition
- Software for running and analyzing experiments
- RTCA SP station or RTCA MP station

The RTCA SP and MP stations fit inside a standard cell culture incubator, ensuring a controlled temperature, humidity, and CO_2 environment. RTCA SP and MP stations accept between one and six 96-well plates (Agilent E-Plate 96), respectively.

Biosensors at the bottom of each well measure cellular impedance. The measured biosensor impedance is expressed in terms of a Cell Index. The CI is a dimensionless value representing the impedance changes of a cell population in contact with the biosensor over time.

The underlying principle of the xCELLigence system is that cells adhere and attach to the sensor surface in the bottom of each cell culture well of the specially engineered E-Plates. In contact with the biosensor, adherent cells behave as insulators, generating resistance to current flow. These small changes in impedance are continuously measured by RTCA instruments, spatially integrated, and expressed over time by the instrument software as CI.

The xCELLigence system also includes the Agilent RTCA dual purpose (DP) instrument, which supports up to three impedance-based plates in two formats: the 16-well E-Plate 16 for cellular assays and the CIM-Plate 16 for cell invasion/migration assays.

Real-time CI monitoring is a reflection of the following basic parameters:

- Cell proliferation kinetics
- Cell size
- Strength of cellular adherence
- Cell viability
- Cell morphology

The RTCA SP, MP, and DP instruments are suitable for addressing how cells respond in a broad spectrum of research fields including drug development, toxicology, cancer biology, medical microbiology, and virology.[10] The impedance-based technology of the xCELLigence system has been shown to work for various applications, such as cell proliferation and cytotoxicity,[10] cell adhesion and spreading,[2] cell culture quality control,[8] receptor tyrosine kinase activation,[3] mast cell activation,[1] and G protein-coupled receptor (GPCR) activation.[9] The most important application in the context of this application note is the detection of NK cell activity in combination with ADCC.[6]

Materials and methods

Cell culture and antibodies

DU145 target cells and NK92 effector cells were obtained from ATCC. NK92 cells were genetically modified to stably overexpress the FcgR III. Both cell lines were grown in a standard cell culture incubator at 37 °C with 5% CO_2. DU145 cells were maintained in RPMI 1640 GlutaMAX media (Gibco) with 10% FCS (Perbio Science), 1% penicillin, and 1% streptomycin (Roche Applied Science). NK92 cells were cultured in MEM alpha medium with L-glutamine (Gibco), 10% FCS, 10% horse serum (Invitrogen), 0.1 mM 2-mercaptoethanol (Gibco), 0.2 mM myo-inositol (Calbiochem), 0.02 mM folic acid (Alfa Aesar), and 10 ng/mL interleukin-2 (Cell Systems). The monoclonal antibody for the ADCC assay was specific for human IGF-1R, binding the human FcgR III with its Fc fragment.

Instrumentation

Impedance measurements and CI determinations were performed using an RTCA MP instrument including a real-time cell analyzer, RTCA MP station, RTCA control unit, and dedicated RTCA Software 1.1.

Real-time cytolytic analysis

DU145 cells were seeded in 200 µL growth medium at a density of 5,000 cells per well onto E-Plate 96. Cell attachment and growth to the logarithmic growth phase were monitored using the RTCA MP instrument. RPMI medium was removed, then NK92 cells in NK cell medium were added to the wells containing DU145 cells. For the antibody studies, NK cells were added at E:T ratios of 3.75:1 or 1.88:1 to DU145 cells that had been pre-incubated with different concentrations of an anti-IGF-1R antibody for 30 minutes. Upon addition of the NK92 effector cells, impedance measurements were made every 15 minutes for up to 88 hours.

Data analysis

The integrated RTCA Software 1.1 displayed the CI values measured in the E-Plate 96 from the time of seeding the DU145 cells, after applying the NK92 cells, and to the end of the experiment at 88 hours. Time- and E:T-dependent proliferation curves were displayed in real time. To quantify the extent of cell lysis at specific time points, CI data were exported to Microsoft Excel, where percentage-of-lysis calculations at specific E:T ratios and antibody concentrations were calculated in reference to the control. IC50 calculations at specific time points or over a period were performed using the RTCA Software 1.1.

Results and discussion

Dynamic monitoring of NK cell-mediated cytolysis of DU145 cells

The DU145 prostate cancer line was seeded as the target at a density of 5,000 cells per well into an E-Plate 96 to assess cell-mediated cytolytic activity. Cells were allowed to attach and proliferate for 20.5 hours. By then, they had reached their logarithmic growth phase as evidenced by dynamic CI monitoring of the DU145 cell population using the RTCA MP instrument every 15 minutes (Figure 1A). At 20.5 hours, real-time measurements were paused, medium was removed, and DU145 cells were exposed to NK92 cells stably overexpressing FcgR III (CD16). These effector cells were added to the target cells in NK cell medium at varying effector-to-target (E:T) cell ratios, ranging from 0.47:1 to 30:1 (a seven-fold doubling dilution series). After the addition of NK92 cells, CI measurements were restarted and changes in proliferation kinetics of the DU145 cells were recorded every 15 minutes for 67.5 hours. There was an initial slight decline in CI values after adding the NK cells due to medium and temperature changes, as shown in Figure 1A. Within one hour after this decline in CI for all treated wells, there was a clear correlation between the number of added effector cells and the concomitant decrease in CI value (Figures 1A and 1B).

Comparing the data between NK92 cells (E) to DU145 (T) cells with E:T ratios from 0.47:1 to 30:1, the CI values showed that, over time, if fewer NK cells were added to the well, fewer target cells were lysed and there was a greater increase of CI. Treatment with NK92 cells at an

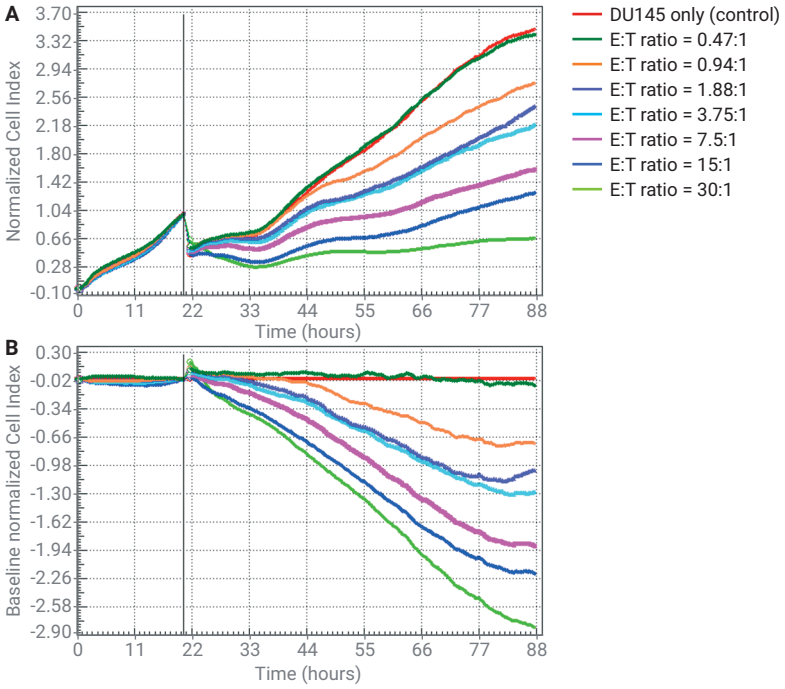

Figure 1. Real-time, label-free monitoring of NK92 cell-mediated cytolysis of DU145 cells. Five thousand DU145 cells per well were seeded in triplicate into an E-Plate 96 and monitored every 15 minutes using the Agilent RTCA MP instrument. At 20.5 hours, NK92 cells were added to the wells at E:T ratios ranging from 0.47:1 to 30:1. The effect of NK cell addition was monitored for 67 hours. A) This plot shows data normalized to the last time point before NK cell addition. B) Same data as above, but with nontreated control cells (red line) defined as baseline. Other curves are plotted in relation to this baseline. Plots were generated using RTCA Software 1.1.

E:T ratio of 30:1 appeared not to allow recovery of the target cells, indicating that most DU145 cells were damaged by NK cell-mediated cytolysis (Figure 1A, light green line). This strong cell lytic effect was observed within a very short incubation time compared to control cells (Figure 1A, red line).

Target cells treated with lower numbers of NK cells (Figure 1A, dark green and other lines) reach distinctive plateau phases of growth at 88 hours, depending on the number of NK cells introduced to the DU145 cells. When comparing plots normalized to either the last time point before NK cell addition (Figure 1A), or to the control cell baseline (Figure 1B), all E:T cell ratios except the lowest E:T ratio of 0.47:1 produced a step-wise differential change in CI values between 33 and 88 hours of the experiment.

Dynamic monitoring of anti-IGF-1R-dependent NK cell-mediated cytolysis of DU145 cells

The insulin-like growth factor 1 receptor (IGF-1R) is a member of the tyrosine kinase receptor family, mediating the effects of IGF-1, a polypeptide similar to insulin. IGF-1 plays an important role during development and growth, and IGF-1 and IGF-1R are implicated in several types of cancer.[7] The development of therapeutic antibodies against IGF-1R, based on their involvement in NK cell-mediated lysis of tumor cells, may be a strategy to treat or prevent cancers.[11]

In the next experiments, cytolytic activity of NK92 cells on DU145 cells was examined in the presence of the anti-IGF-1R monoclonal antibody, which binds to both extracellular human IGF-1R (by its Fab fragments) and the Fcg receptor III (FcgR III) on NK92 cells (by its Fc fragment).Again, 5,000 DU145 cells were seeded into each well of an E-Plate 96, and logarithmic growth was monitored every 15 minutes for 20.5 hours. NK cells in NK cell medium were added at E:T ratios of 3.75:1 (Figure 2A) and 1.88:1 (Figure 3A). DU145 target cells were pre-incubated with different amounts of anti-IGF-1R for 30 minutes, resulting in final concentrations between 0.1 and 100 µg/mL. The effect of NK cells without antibody (untreated control), as well as the effect of the varying amounts of antibody were recorded every 15 minutes for 67.5 hours.

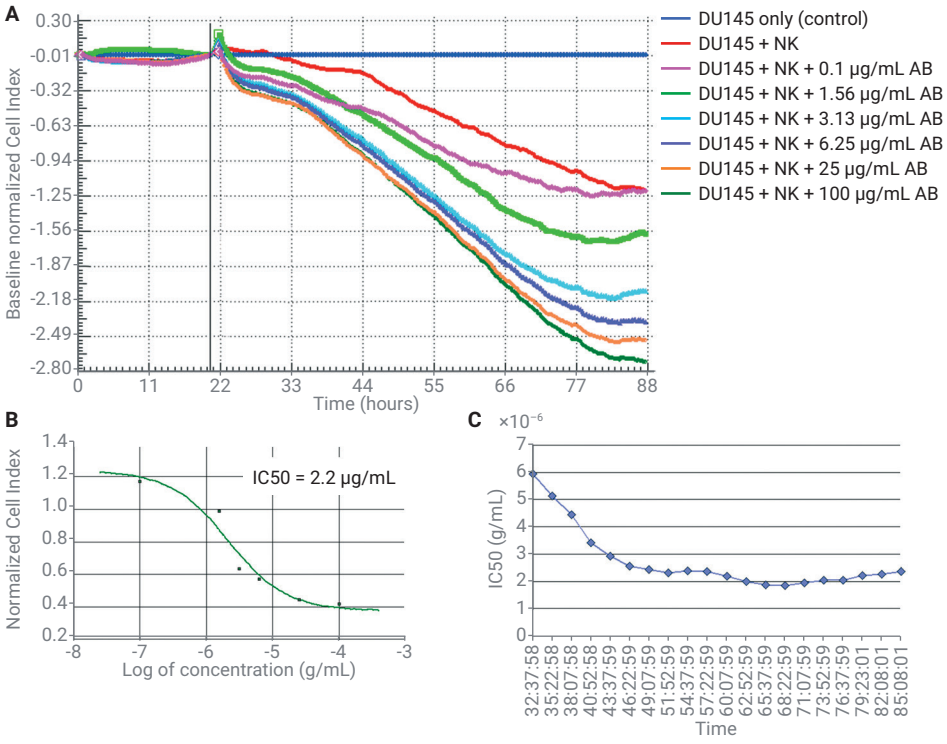

Figure 2. Effect of the anti-IGF-1R antibody on NK cell-mediated cytolysis of DU145 cells (E:T ratio = 3.75:1). Five thousand DU145 cells per well were seeded in triplicate into an Agilent E-Plate 96 and monitored every 15 minutes using the Agilent RTCA MP instrument. After 20.5 hours, NK92 cells were added to the wells at an E:T ratio of 3.75:1. Target cells were pre-incubated in advance with different concentrations of anti-IGF-1R antibody for 30 minutes. The effect of NK cell and anti-IGF-1R addition was monitored for 67 hours. A) Plot of normalized CI values of the entire course of the experiment (88 hours). Data are normalized to the last time point before NK cell addition and curves are plotted with control wells (DU145 cells only) set as baseline. B) Calculation of IC50 after 60 hours. C) Calculation of time-dependent IC50 from nine hours after NK cell addition to the end of the experiment at 88 hours. All plots were generated using the RTCA Software 1.1.

In comparison to nontreated control cells, NK cells induced a moderate decline in CI values on DU145 cells without antibody pretreatment (red line). This was due to to NK-mediated cytolysis of DU145 cells (compare controls to antibody-treated wells in Figures 2A and 3A). DU145 pretreatment with antibody clearly produced a concentration-dependent decrease in impedance and CI values (summarized in Table 1). CI values of anti-IGF-1R-treated DU145 cells were lower than that of DU145 cells without antibody pretreatment, indicating that the presence of the antibody increased the cytotoxic effect of the NK cells (Figures 2A and 3A, Table 1).

Table 1. NK cell-mediated cytolysis of DU145 cells 48 hours after NK cell addition and DU145 cell pretreatment with increasing amounts of IGF-1R antibody. NK92 cells were added to DU145 cells at different effector cell:target cell (E:T) ratios. The percentage of cytolysis was calculated based on the normalized CI values 48 hours after DU145 antibody pretreatment and NK cell addition. The last time point before addition of the NK92 cells was chosen for normalization of data.

E:T Ratio	Anti-IGF1R Antibody (µg/mL)	Cytolysis (%) After 48 Hours
0.47:1	–	0
0.94:1	–	21
1.88:1	–	35
	0.1	36
	0.78	42
	6.25	47
	25	52
	100	58
3.75:1	–	37
	0.1	40
	1.56	54
	3.13	69
	6.25	73
	25	79
	10	82
7.5:1	–	54
15:1	–	66
30:1	–	80

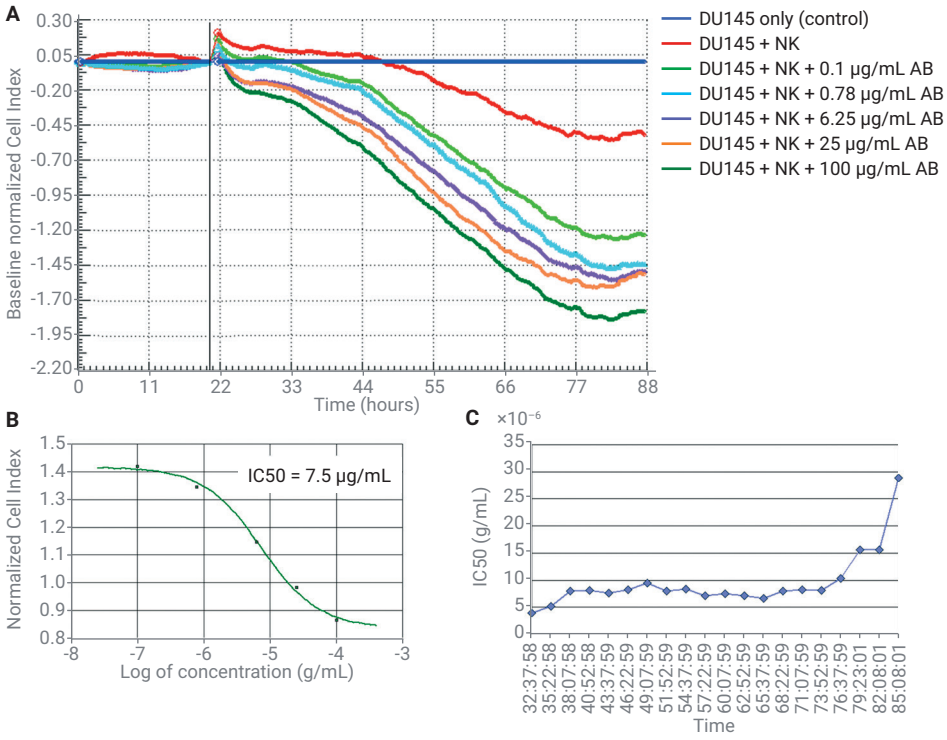

Figure 3. Effect of the anti-IGF-1R antibody on NK cell-mediated cytolysis of DU145 cells (E:T ratio = 1.88:1). Five thousand DU145 cells per well were seeded in triplicate into an Agilent E-Plate 96 and monitored every 15 minutes using the Agilent RTCA MP instrument. After 20.5 hours, NK92 cells were added to the wells at an E:T ratio of 1.88:1. Target cells were pre-incubated in advance with different concentrations of anti-IGF-1R for 30 minutes. The effects of NK cell and anti-IGF-1R pretreatment were monitored for 67 hours. A) Plot of normalized CI values of the entire course of the experiment (88 hours). Data are normalized to the last time point before NK cell addition and curves are plotted with control wells (DU145 cells only) set as baseline. B) Calculation of IC50 after 60 hours. C) Calculation of time-dependent IC50 from nine hours after NK cell addition to the end of the experiment at 88 hours. All plots were generated using the RTCA Software 1.1.

Anti-IGF-1R was hypothesized to increase the interaction between DU145 and NK cells by cross-linking overexpressed FcgR III on NK92 cells. The CI findings showed that a clear antibody dose-response dependency and the degree of NK92-mediated cell lysis is also a function of the E:T ratio: The more NK cells administered, the stronger the additive effect of the antibody (Table 1). In contrast, DU145 cell treatment with anti-IGF-1R in the absence of NK92 cells showed no reduction in CI values compared to control levels. In fact, antibody amounts over 6.25 µg/mL produced a slight increase in CI values, and corresponding DU145 proliferation curves clearly run above the curve representing nontreated control cells (Figure 4).

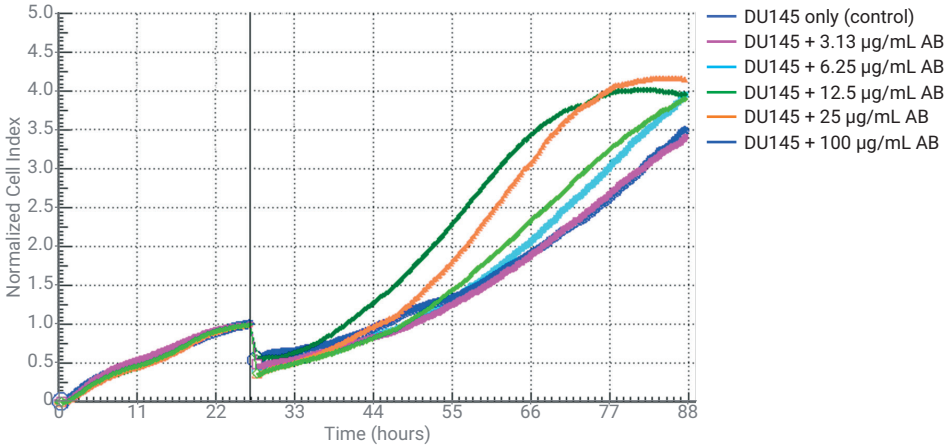

Figure 4. Real-time, label-free monitoring of the effect of anti-IGF-1R antibody on DU145 cells in the absence of NK92 effector cells. Five thousand DU145 cells per well were seeded in triplicate into an Agilent E-Plate 96 and monitored every 15 minutes using the Agilent RTCA MP instrument. After 27 hours, anti-IGF-1R was added to the wells at the indicated concentrations. The effect of the anti-IGF-1R addition was monitored for 60 hours. Plotted CI values were normalized to the last time point before the addition of antibody. Plots were generated using the RTCA Software 1.1.

Calculation of half inhibitory (IC50) anti-IGF-1R concentrations

To further quantify the effects of the anti-IGF-1R antibody on the NK92 and DU145 cytolytic cell-cell interaction, normalized CI values obtained from the antibody dose-responses were used to calculate IC50 values using the RTCA Software 1.1. Data obtained from experiments using either 3.75:1 or 1.88:1 NK92:DU145 (E:T) cell ratios were used to calculate IC50 values of 2.2 and 7.5 µg/mL, respectively (Figures 2B and 3B). The IC50 value was significantly higher for the lower number of NK cells added. This indicated that approximately 3.5 times more antibody was needed to induce the same extent of target cell lysis when only half of the NK cell amount was administered.

Time-dependent IC50 values for more than 50 hours of the experiment were calculated using another tool in the RTCA Software 1.1, showing the excellent reproducibility of IC50 values over this long period (Figures 2C and 3C).

Conclusion

The present study shows the feasibility of using the xCELLigence system to monitor both NK cell-mediated tumor cell cytolysis and ADCC in a label-free, noninvasive manner, corroborating earlier findings by Glamann and Hansen.[6] The experiments highlighted in this application note show both the quantitative effect of adding different amounts of NK92 cells, as well as the additive cytolytic effect of introducing an NK92-dependent anti-IGF-1R monoclonal antibody.

The xCELLigence system is ideal for directly monitoring ADCC without the need for labeling the target cells or using a chemical reporter. The entire cytolytic process can now be measured using impedance electronic sensing to detect both expected and unexpected cellular responses, an impossible task for previously described conventional endpoint assay formats.

References

1. Abassi, Y. A. *et al.* Label-Free, Real-Time Monitoring of IgE-Mediated Mast Cell Activation on Microelectronic Cell sensor Arrays. *J. Immunol. Methods* **2004**, *292*, 195–205.
2. Atienza, J. M. *et al.* Label-Free and Real-Time Cell-Based Kinase Assay for Screening Selective and Potent Receptor Tyrosine Kinase Inhibitors Using Microelectronic Sensor Array. *J. Biomol. Screen*. **2006**, *11*, 634–643.
3. Atienza, J. M. *et al.* Dynamic Monitoring of Cell Adhesion and Spreading on Microelectronic Sensor Arrays. *J. Biomol. Screen*. **2005**, *10*, 795–805.
4. Brunner, K. T. *et al.* Quantitative assay of the Lytic Action of Immune Lymphoid Cells on 51-Cr-Labeled Allogeneic Target Cells In Vitro; Inhibition By Isoantibody and by Drugs. *Immunology* **1968**, *14*, 181–196
5. De Meyer, K. *et al.* Morphometric Analysis of Cytolysis in Cultured Cell Monolayers: a Simple and Versatile Method for the Evaluation of the Lytic Activity and the Fate of LAK cells. *J. Immunol. Methods* **2003**, *277*, 193–211.
6. Glamann, J.; Hansen, A. J. Dynamic Detection of Natural Killer Cell-Mediated Cytotoxicity and Cell Adhesion by Electrical Impedance Measurements. *Assay Drug Dev. Technol*. **2006**, *4*, 555–63.
7. Hartog, H. *et al.* The Insulin-Like Growth Factor 1 Receptor in Cancer: Old Focus, New Future. *Eur. J. Cancer* **2007**, *43*, 1895–1904.
8. Kirstein, S. L. *et al.* Live Cell Quality Control and Utility of Real-Time Cell Electronic Sensing for Assay Development. *Assay Drug Dev. Technol*. **2006**, *4*, 545–553.
9. Yu, N. *et al.* Real Time Monitoring of Morphological Changes in Living Cells by Electronic Cell Sensor Arrays: an Approach to Study G Protein-Coupled Receptors. *Anal. Chem*. **2006**, *78*, 35–43.
10. Solly, K.; Wang, X. *et al.* Application of Real-Time Cell Electronic Sensing (RT-CES) Technology to Cell-Based Assays. *Assay Drug Dev. Technol*. **2004**, *2*, 363–372.
11. Stagg, J.; Smyth, M. J. NK Cell-Based Immunotherapy. *Drug News Perspect.* **2007**, *20*, 155–163.

Printed in the USA, November, 1, 2019
5994-1063EN
AN 10
DE.5648611111

Contact OLS OMNI Life Science - Your Partner in Cell Research

OLS OMNI Life Science GmbH & Co. KG
Karl-Ferdinand-Braun-Straße 2
28359 Bremen, Germany
Phone: +49 421 27 61 69 0
info@ols-bio.de • www.ols-bio.com

OLS OMNI Life Science GmbH
Laufenstraße 90
4053 Basel, Switzerland
Phone: +49 421 27 61 69 0
info@ols-bio.ch • www.ols-bio.com

OLS® OMNI Life Science